# Analysis of model replication origins in *Drosophila* reveals new aspects of the chromatin landscape and its relationship to origin activity and the prereplicative complex

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**ABSTRACT** **Epigenetic regulation exerts a major influence on origins of DNA replication during development. The mechanisms for this regulation, however, are poorly defined. We showed previously that acetylation of nucleosomes regulates the origins that mediate developmental gene amplification during *Drosophila* oogenesis. Here we show that developmental activation of these origins is associated with acetylation of multiple histone lysines. Although these modifications are not unique to origin loci, we find that the level of acetylation is higher at the active origins and quantitatively correlated with the number of times these origins initiate replication. All of these acetylation marks were developmentally dynamic, rapidly increasing with origin activation and rapidly declining when the origins shut off and neighboring promoters turn on. Fine-scale analysis of the origins revealed that both hyperacetylation of nucleosomes and binding of the origin recognition complex (ORC) occur in a broad domain and that acetylation is highest on nucleosomes adjacent to one side of the major site of replication initiation. It was surprising to find that acetylation of some lysines depends on binding of ORC to the origin, suggesting that multiple histone acetyltransferases may be recruited during origin licensing. Our results reveal new insights into the origin epigenetic landscape and lead us to propose a chromatin switch model to explain the coordination of origin and promoter activity during development.**

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## INTRODUCTION

Efficient duplication of large eukaryotic genomes requires that DNA replication initiate from multiple origins. In multicellular eukaryotes, however, it remains largely unknown how certain genomic loci are selected to be active origins of DNA replication; a DNA consensus for origins has yet to emerge. Moreover, the selection of origin loci and their time of initiation during S phase change during development (Mechali, 2010). Current evidence suggests that chromatin modifications play a major role in the developmental regulation of origins. Here we investigate the epigenetic regulation of the well-defined model origins that mediate developmental gene amplification during *Drosophila* oogenesis.

The proteins and mechanisms that regulate origins during the cell cycle are conserved in eukaryotes (Remus and Diffley, 2009). During early G1 phase, a prereplicative complex (preRC) assembles onto origins, preparing them for replication (Diffley *et al.*, 1995). Assembly of the preRC begins with the binding of the origin recognition complex (ORC) to DNA, which recruits Cdc6 and Cdt1, both of which are required for loading of the minichromosome maintenance (MCM) helicase complex onto origin DNA (Yan *et al.*, 1991; Bell *et al.*, 1993; Chong *et al.*, 1995; Cocker *et al.*, 1996; Maiorano *et al.*, 2000; Nishitani *et al.*, 2000). Once the preRC is assembled, the origin is "licensed" but has yet to initiate DNA replication. During different times of S phase, subsets of these preRCs are activated by cyclin-dependent kinase (CDK) and Cdc7 kinase, which results in the association of other proteins to the origin, the initiation of DNA replication, and departure of preRC proteins from the origin (Labib, 2010). The preRC is prevented from reassembling onto origins until

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Abbreviations used: ACE1, amplification control element on chromosome X; ACE3, amplification control element on chromosome 3; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; cp, chorion protein; DAFC, *Drosophila* Amplicon in Follicle Cells; GFP, green fluorescent protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; MCM, minichromosome maintenance; ORC, origin recognition complex; preRC, prereplicative complex.





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the next cell cycle, thereby ensuring that the genome duplicates once per cell division (Arias and Walter, 2007). Defects in preRC regulation can result in DNA damage, genome instability, human developmental abnormalities, and cancer (Lengronne and Schwob, 2002; Hook *et al.*, 2007; Liontos *et al.*, 2007; Mehrotra *et al.*, 2008; Green *et al.*, 2010; Bicknell *et al.*, 2011; Guernsey *et al.*, 2011).

Despite the conserved mechanism of origin regulation, it remains unclear in multicellular eukaryotes how certain regions of the genome are selected to be sites of preRC assembly and activation (Mechali, 2010). Although only a handful of metazoan origins have been analyzed both genetically and molecularly, the emerging theme is that most are large and modular, with functional DNA elements spread over several to tens of kilobases. Genomic approaches have mapped thousands of preRC-binding sites and active origins within several multicellular genomes, including *Drosophila* and human (Cadoret, 2008; Sequeira-Mendes, 2009; Gilbert, 2010; Hansen *et al.*, 2010; Karnani *et al.*, 2010; MacAlpine *et al.*, 2010). Nevertheless, a strict DNA consensus for origins has yet to emerge (Aladjem, 2007). In fact, ORC does not display sequence specificity for DNA binding in vitro, although it does prefer AT-rich sequences and negatively supercoiled DNA (Bielinsky *et al.*, 2001; Chesnokov *et al.*, 2001; Vashee *et al.*, 2003; Remus *et al.*, 2004). In contrast, there are thousands of preferred sites for ORC binding and replication initiation on native chromosomes, whose location and S phase timing change during development (Huberman, 1968; Lima-de-Faria and Jaworska, 1968; Mechali, 2010). An important remaining question, therefore, is what specifies genomic loci to be preferred sites for preRC assembly and activation in different cells.

We have been studying developmental gene amplification in the follicle cells of the *Drosophila* ovary as a model for origin structure and regulation in a developmental context. Amplification is a local increase in gene copy number due to site-specific rereplication from origins at two loci that encode eggshell (chorion) proteins on the X (*Drosophila* Amplicon in Follicle Cells-7F, DAFC-7F) and third chromosome (DAFC-66D) and at four other, recently identified loci (DAFC-22B, DAFC-30B, DAFC-34B, and DAFC-62D), some of which encode proteins that assist vitelline membrane and eggshell synthesis (Spradling, 1981; Calvi *et al.*, 1998; Calvi, 2006; Claycomb *et al.*, 2004; Claycomb and Orr-Weaver, 2005; Kim *et al.*, 2011). The amplicon origins are bound by a preRC and regulated by both CDK2 and CDC7 kinases (Calvi *et al.*, 1998; Austin *et al.*, 1999; Landis and Tower, 1999; Whittaker *et al.*, 2000; Schwed *et al.*, 2002). The amplicon origins therefore share many attributes with origins that govern normal genomic replication, and the analysis of these model origins has provided new insights into origin structure and regulation (Claycomb and Orr-Weaver, 2005; Calvi, 2006).

We previously showed that nucleosome acetylation at the amplicon origins contributes to their developmental specificity and efficiency (Aggarwal and Calvi, 2004), consistent with results from others (Lewis *et al.*, 2004; Hartl *et al.*, 2007). A general relationship between active origins and histone acetylation has been supported by genome-wide mapping and the detailed analysis of a few origins in multiple organisms, although this correlation is not perfect (Kim *et al.*, 2003; Danis *et al.*, 2004; Cadoret, 2008; Hiratani *et al.*, 2008, 2010; Schwaiger *et al.*, 2009, 2010; Bell *et al.*, 2010; Eaton *et al.*, 2010a; Gilbert, 2010; MacAlpine *et al.*, 2010). Experiments from yeast to human have also identified some of the histone acetyltransferases (HATs) and histone deacetylases (HDACs) that can influence preRC assembly and the time of origin activation in S phase (Iizuka and Stillman, 1999; Iizuka *et al.*, 2009; Vogelauer *et al.*, 2002; Aggarwal and Calvi, 2004; Stedman *et al.*, 2004; Jorgensen *et al.*, 2007; Crampton *et al.*, 2008; Fox and Weinreich, 2008; Wu and Liu, 2008; Schwaiger *et al.*, 2009; Miotto and Struhl, 2010; Wong *et al.*, 2010). Another attribute of origins in eukaryotes is that they coincide with "nucleosome free regions," which are now known to represent domains of dynamic nucleosome–DNA association (Simpson, 1990; Lipford and Bell, 2001; Urnov *et al.*, 2002; Mito *et al.*, 2007; Eaton *et al.*, 2010a, 2010b; MacAlpine *et al.*, 2010; Muller *et al.*, 2010). Current evidence suggests, therefore, that both nucleosome modification and position locally influence origin activity. Despite these advances, the molecular mechanisms by which chromatin influences origin function remain poorly defined.

Here we extend our analysis of the epigenetic regulation of the model amplification origins. Our results reveal new aspects of the origin epigenetic landscape and the relationship connecting histone acetylation, ORC binding, and origin activity.

## RESULTS

### Dynamic acetylation of multiple histone lysine residues is associated with DAFC-66D developmental timing

Site-specific rereplication at six origin loci results in developmental gene amplification during *Drosophila* oogenesis These origins become active in somatic follicle cells at precisely stage 10B of oogenesis, a time when other origins are not active and genomic replication has ceased, and therefore represents an extreme form of origin developmental specificity (Calvi *et al.*, 1998; Claycomb and Orr-Weaver, 2005). Previous analysis indicated that the N-terminal tails of histones H3 and H4 are hyperacetylated at the origins, and this hyperacetylation stimulates origin activity (Aggarwal and Calvi, 2004; Hartl *et al.*, 2007). These experiments used antibodies against polyacetylated H3 and H4, as well as those raised against specific acetylated lysine residues. It is now widely appreciated that some commercially available antibodies are not as specific as once believed (Egelhofer *et al.*, 2011). Therefore, to further explore the mechanism of origin epigenetic regulation, we used antibodies that have been recently validated by the modENCODE research consortium and, where possible, used multiple antibodies from different suppliers against the same modification (Egelhofer *et al.*, 2011).

To determine whether these antibodies label active amplicon origins, we used them for immunofluorescence labeling of fixed ovaries. These ovaries were first incubated in bromodeoxyuridine (BrdU) to detect exclusive replication from the amplicon origins, which appears as distinct foci of BrdU incorporation in follicle cell nuclei beginning in stage 10B (Calvi *et al.*, 1998; Calvi and Spradling, 2001). The acetylated histone antibodies displayed a general labeling throughout the nucleus up until stage 10A, just before the start of amplification. Figure 1 shows the results for anti-H4K16Ac and anti-H4K12Ac labeling (Figure 1, A–A″ and D–D″). From stage 10B to stage 11/12, however, many of the acetylated histone antibodies strongly labeled the amplicon BrdU foci, in addition to there being a lower level of nucleus-wide labeling (Table 1 and Figure 1, B–B″ and E–E″). Although multiple amplicon BrdU foci were often labeled, the most prominent labeling corresponded to the DAFC-66D locus, which amplifies to the highest DNA copy number (~64-fold) and is the largest BrdU focus (Figure 1, B–B″ and E–E″; Calvi *et al.*, 1998). Consistent with our previous results, the labeling of amplicon foci by acetylated histone antibodies often appeared as a bar in the center of a larger bar of BrdU, which represents waves of replication forks emanating bidirectionally outward from the origins (Figure 1, B–B″; Aggarwal and Calvi, 2004). This pattern suggests that acetylation occurs near the center of the replicon and does not simply represent acetylation of newly deposited nucleosomes behind the

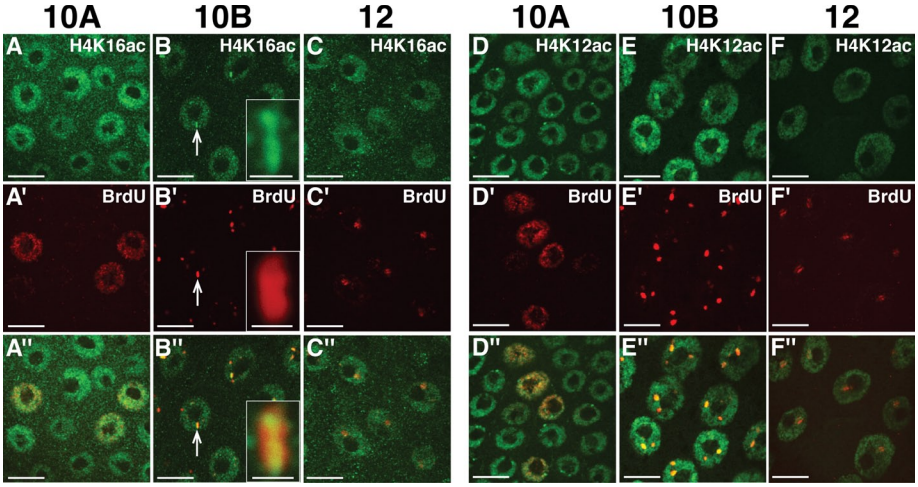


FIGURE 1: Hyperacetylation of H4K16 and H4K12 at active amplification origins. Follicle cells were colabeled with α-BrdU (A′–F′) and α-H4K16ac (A–C) or α-H4K12ac (D–F) from oogenesis stages 10A (A–A″ and D–D″), 10B (B–B″ and E–E″), and 12 (C–C″ and F–F″). Merged images are shown in A″–F″. Arrows in B–B″ indicate a single amplicon focus corresponding to DAFC-66D, which is enlarged in the insets. Scale bars, 10 μm; inset scale bars, 1 μm.

replication forks. Although other genomic loci in stage 10B/11 follicle cells were labeled by acetylated histone antibodies, the relative level of fluorescence labeling at amplicon loci was much greater, which we previously showed is not an artifact of increased DNA copy number during amplification (Aggarwal and Calvi, 2004). Labeling for these different types of histone acetylation rapidly disappeared at DAFC-66D in early stage 12, a time when the origin shuts off, ORC departs, but forks continue to migrate outward (Figure 1, C–C″ and F–F″; Royzman *et al.*, 1999; Aggarwal and Calvi, 2004). This further argues that this acetylated histone labeling does not correspond to chromatin assembly behind the replication fork. Overall, the immunofluorescence results suggest that nucleosomes near the origins are hyperacetylated on multiple lysine residues during developmental stages when the origin is active.

We next turned to chromatin immunoprecipitation (ChIP) to analyze chromatin modification at the origins, a method that is of higher resolution and more sensitive than immunofluorescence labeling. It has the added advantage that the signal is normalized to input DNA, and, therefore, acetylation per chromatin fiber can be compared among amplicon loci that amplify to different DNA copy numbers. We also quantified the relative level of acetylation at the origins by normalizing the ChIP–real-time quantitative PCR (qPCR) signal to different nonorigin control loci measured in parallel (Supplemental Figure S1). To evaluate the developmental dynamics of chromatin modification, thousands of egg chambers from different stages of oogenesis were mass isolated, followed by hand selection of specific developmental stages and purification of follicle cell nuclei. We initially focused on DAFC-66D because it is the best-characterized amplicon origin. At this origin, the 320–base pair ACE3 and the 840–base pair Ori-β are both necessary and sufficient for origin function and contain preferred binding sites for the ORC (Figure 2A; Orr-Weaver *et al.*, 1989; Austin *et al.*, 1999; Zhang and Tower, 2004; Calvi, 2006). Although both of these elements bind ORC, Ori-β is the preferred site of replication initiation (~80% of the time; Delidakis and Kafatos, 1989; Heck and Spradling, 1990; Zhang and Tower, 2004). Each of these origin elements is immediately upstream of chorion protein genes (cp18 and cp15) but can be functionally separated from its promoters (Figure 2A; (Orr-Weaver and Spradling, 1986). Initiation from the DAFC-66D origin occurs from stage 10B to early stage 12 (~7 h), after which the origin shuts off and nearby promoters turn on in stage 12/13 (Griffin-Shea *et al.*, 1982; Orr *et al.*, 1984; Royzman *et al.*, 1999).

ChIP-qPCR showed that multiple lysine residues are hyperacetylated at DAFC-66D, confirming the results of immunofluorescence labeling. Similar to the immunofluorescence results, other loci in follicle cells were immunoreactive to the acetylated histone antibodies in ChIP assays. The level of hyperacetylation at DAFC-66D, however, was ~20–60 times greater relative to two other nonorigin loci, using antibodies against different acetylated lysines in the tail of histone H4 (K5, K8, K12, and K16; Table 1, Figure 2, B–E, and Supplemental Figure S1). We previously reported hyperacetylation on lysines in the histone H3 tails at the amplicon and confirmed this result using antibodies against H3K9/14Ac (Aggarwal and Calvi, 2004; Table 1). We also obtained evidence for acetylation on lysine 56 within the core of histone H3, which was the most enriched of any modification in stage 10 (Figure 2F). Control ChIP experiments with antibodies against total histone H3 did not give evidence for enrichment at the amplicons (Supplemental Figure S2). ChIP with antibodies against marks associated with repressive chromatin (e.g., H3K27me3) also did not give evidence for enrichment at the DAFC-66D origin (Supplemental Figure S3A). Analysis of stages before, during, or after the origin is active showed that all the acetylation marks had similar developmental timing. Acetylation on all residues was low in early developmental stages (stage ≤8) when the origin is inactive, peaked in stage 10/11 when the origin was active, and then rapidly declined in stages 12 and 13 when ORC departs and the origin shuts off (Figure 2, B and F). These results reveal that multiple types of nucleosome hyperacetylation are temporally correlated with developmental origin activity during oogenesis.

| Antibody | Immunostaining[a] | ChIP[b] |
|---|---|---|
| H4K5ac | +[c] | + |
| H4K8ac | +[c] | + |
| H4K12ac (Active Motif) | + | + |
| H4K12ac (Millipore) | + | + |
| H4K16ac (Active Motif) | + | + |
| H4K16ac (Millipore) | f | + |
| H3K9/14ac | n.d. | + |
| H3K56ac | + | + |
| H3K4me3 | f | – |
| H3K27me3 | – | – |

[a]+, BrdU foci labeled; –, BrdU foci not labeled; f, antibody failed to label fixed tissue; n.d.: not determined.
[b]+, enriched over control loci; –, not enriched.
[c]From Aggarwal and Calvi (2004).

TABLE 1: Summary of immunostaining and ChIP results for histone modifications at DAFC-66D in stage 10B follicle cells.

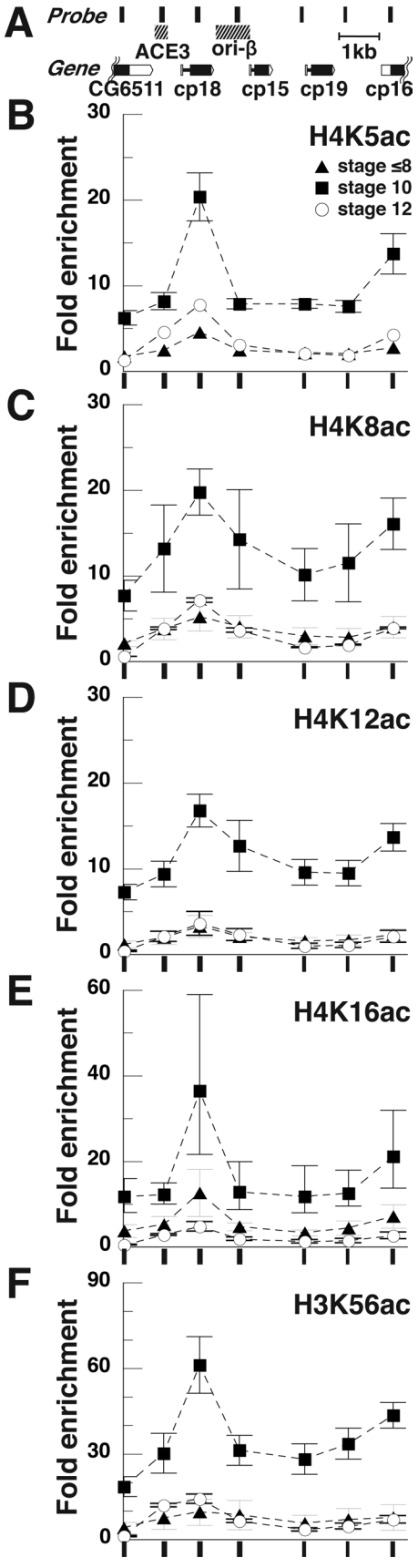


FIGURE 2: Developmentally dynamic acetylation of nucleosomes on multiple lysine residues correlates with activity of the DAFC-66D origin. (A) Diagram of DAFC-66D locus. Hatched boxes represent essential elements ACE3 and Ori-β (middle); the location of ChIP-qPCR products is shown above, and the position of four chorion protein (cp) genes and a flanking gene is shown below. (B–F) ChIP-qPCR analysis with the indicated antibodies using follicle cells from oogenesis stages 8 and earlier (▲), stage 10 (■), and stage 12 (○). The positions of the PCR products are shown on the *x*-axis and aligned with the map in A; the *y*-axis represents the fold enrichment of ChIP-qPCR signal over a control region in the genome (at cytogenetic region 64A), and the error bars represent the range of data from two or three biological replicates.

### Nucleosome acetylation extended across the DAFC-66D origin, with a peak adjacent to the major site of replication initiation

We had used multiple primer pairs for ChIP-qPCR to define the profile of acetylation at DAFC-66D and its relationship to functional origin regions. Hyperacetylation of multiple lysines was not restricted to the essential 320–base pair ACE3 and 840–base pair Ori-β but was at least five- to15-fold enriched for the different antibodies over a 7-kb region encompassing the four chorion protein genes (Figure 2). Although nucleosome acetylation was significantly enriched across this domain, a distinct peak of acetylation on multiple lysines was detected between ACE3 and Ori-β in the body of the cp18 gene (Figure 2). This peak of acetylation is not an artifact of copy number or ChIP method, because all signals are normalized to input and a nonorigin control locus. The data suggest that nucleosomes are hyperacetylated in a domain at the DAFC-66D locus and that within the population of follicle cells acetylation is most frequent on nucleosomes adjacent to one side of the major site of replication initiation in Ori-β.

### Nucleosome acetylation is a general property of amplicon origins and quantitatively correlates with origin activity

To determine whether hyperacetylation of multiple histone lysine residues is a general attribute of amplicon origins, we analyzed the other amplicons by ChIP-qPCR. We first analyzed DAFC-7F on the X chromosome, which encodes five chorion proteins and amplifies to ~16-fold (Figure 3A; Spradling, 1981). Similar to DAFC-66D, nucleosomes were hyperacetylated on multiple lysines extending across a domain at the DAFC-7F origin. There were two peaks of acetylation—one in the body of the cp36 gene and the other in cp38 (Figure 3). The latter peak corresponds to ACE1, a region that has been shown to bind ORC and that is required in *cis* for origin function (Spradling *et al.*, 1987; Austin *et al.*, 1999). The developmental timing of acetylation at DAFC-7F was also similar to that at DAFC-66D. Acetylation was low in early stages of oogenesis, greatly increased when the origin is active in stage 10, and declined later when the origin shuts off (Figure 3, B–F).

We further analyzed four other loci that have been reported to amplify to low levels in follicle cells in late oogenesis (DAFC-22B, DAFC-30B, DAFC-34B, and DAFC-62D; Claycomb *et al.*, 2004; Kim *et al.*, 2011). We found that nucleosomes at these other amplicons were also dynamically acetylated on multiple lysine residues when these origins became active in stage 10 of oogenesis (Figure 4). The one exception was DAFC-22B, which was not significantly hyperacetylated relative to the control locus (Figure 4). Although at first this seemed to challenge the generality of nucleosome hyperacetylation at active amplicon origins, the Orr-Weaver lab recently reported that the DAFC-22B origin is not active in the wild-type strain that we and the modENCODE project used for ChIP, Oregon-R$^{modENCODE}$ (Kim *et al.*, 2011). These results indicate, therefore, that acetylation on multiple histone lysine residues is a general attribute of amplicon origins when they are developmentally activated in late oogenesis.

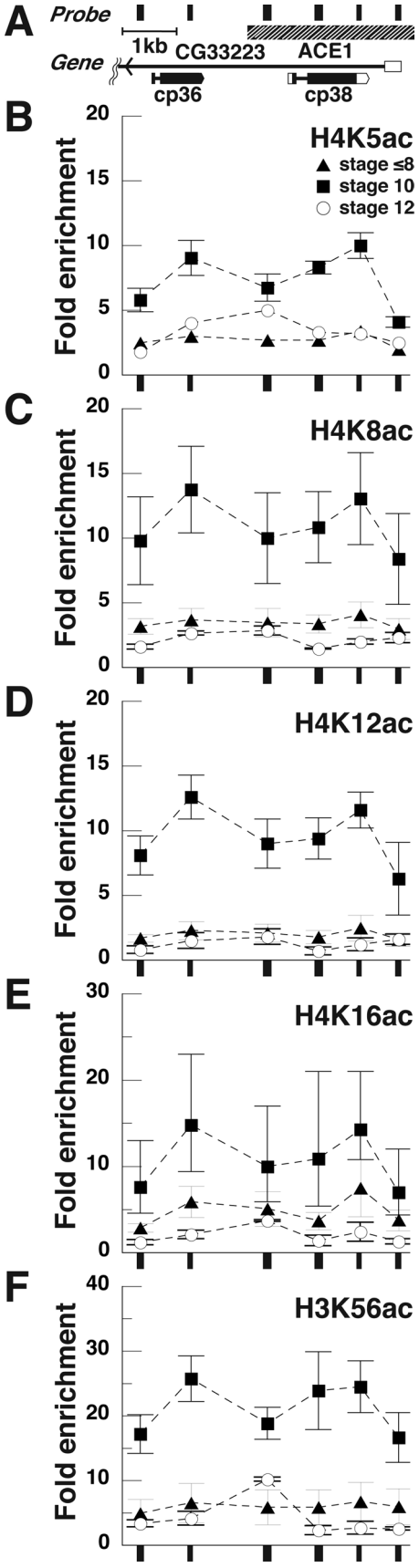


FIGURE 3: Nucleosomes are dynamically acetylated on multiple lysine residues at the DAFC-7F origin. (A) Diagram of DAFC-7F locus and qPCR probes. The hatched box represents the origin ACE1 element. (B–F) ChIP-qPCR analysis using the indicated antibodies on follicle cells from oogenesis stages 8 and earlier (▲), stage 10 (■), and stage 12 (○). PCR products are shown below each panel and aligned with A. The error bars represent the range of data from two or three biological replicates.

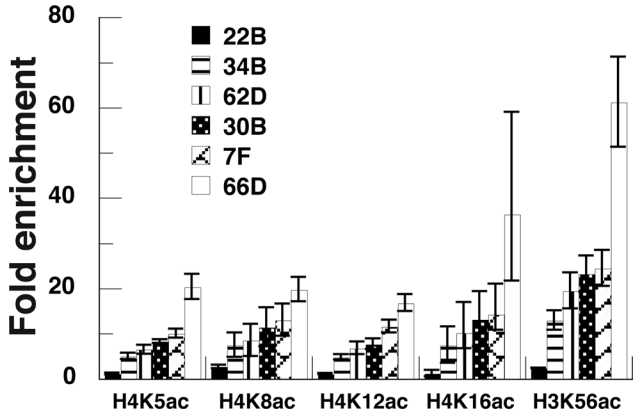


FIGURE 4: Histone acetylation levels correlate with amplicon origin efficiency. ChIP-qPCR results for six amplicon loci (DAFC-22B, -34B, -62D, -30B, -7F, and -66D) from stage 10 follicle cells using the antibodies indicated. The error bars represent the range of data from two or three biological replicates.

Another relationship between acetylation and origin activity emerged from comparison of the different amplicons; the level of hyperacetylation was greater for loci that amplify to higher final DNA copy number. The fold enrichment of all acetylation marks was higher for DAFC-66D (~64-fold amplified) than DAFC-7F (~16-fold amplified; Figure 4; Spradling, 1981). This trend continued, with acetylation levels being even lower for DAFC-62D (~3- to 6-fold amplified) and DAFC-30B (approximately fourfold amplified; Figure 4; Claycomb *et al.*, 2004). The extreme is DAFC-22B, which was not acetylated or amplified in the Oregon-R$^{modENCODE}$ strain (Figure 4). Again, this trend is not an artifact of different DNA copy number, because all ChIP-qPCR is normalized to input DNA, and antibodies against unmodified histone H3 did not give evidence for enrichment (Supplemental Figures S1 and S2). Overall, the results indicated that, whereas acetylation is not unique to the origin loci, the level of acetylation on multiple lysines is much higher at the active origins and quantitatively correlates with the number of times different origins initiate replication.

### Histone acetylation is not dependent on origin activation

The foregoing results suggested that multiple lysine residues are rapidly acetylated when amplicon origins become active and then are rapidly deacetylated when the origin shuts off and neighboring promoters turn on. ChIP for a marker of active transcription, H3K4me3, confirmed that neighboring promoters were not active during stages when the origin was active and highly acetylated (Supplemental Figure S3B). This suggests that acetylation at the origins does not represent activation of adjacent promoters. A possible caveat, however, is that hyperacetylation may simply represent modification on newly deposited nucleosomes behind the fork and therefore is a consequence, but not cause, of origin activity. Arguing against this interpretation is the observation that acetylation rapidly declined at DAFC-66D in stages 12 and 13, a time when the origin is quiescent but replication forks continue to migrate (Figure 1, C–C″ and F and F″, Figure 2, and data not shown; Claycomb *et al.*, 2002; Aggarwal and Calvi, 2004).

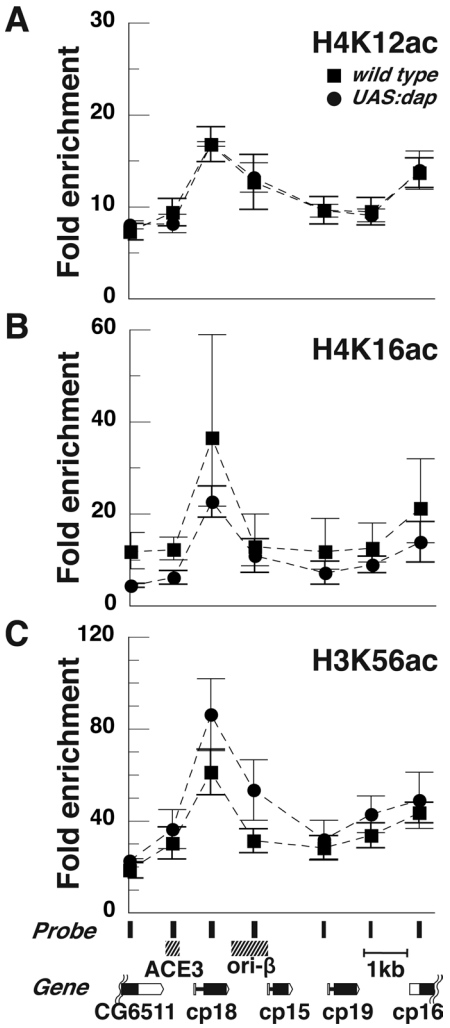


FIGURE 5: Acetylation is not dependent on replication initiation. Acetylation level at DAFC-66D was compared between wild-type follicle cells and those in which initiation was inhibited by expression of *UAS:dap*. (A–C) ChIP-qPCR results using the indicated antibodies on stage 10 follicle cells from wild-type Oregon R (■) and *c323GAL4/+;UAS:dap/+* (●) flies. The Oregon R data from Figure 2 were graphed for comparison. Drawn to scale for the DAFC-66D locus shown below. The error bars represent the range of data from two or three biological replicates.

To test rigorously whether acetylation simply represents nucleosome deposition behind the fork, we blocked the initiation step of DNA replication and measured acetylation at the DAFC-66D amplicon by ChIP-qPCR. We previously showed that overexpression of the cyclin E/CDK2 inhibitor *dacapo* (*dap*) severely inhibits gene amplification, resulting in a thin eggshell phenotype (Calvi *et al.*, 1998). We overexpressed *UAS:dap* specifically in late-stage follicle cells using the c323GAL4 driver, which resulted in reduced amplification that was undetectable by BrdU incorporation in all but a few follicle cell nuclei (Calvi *et al.*, 1998). Quantification of DNA copy number at DAFC-66D and DAFC-7F in stage 10 and stage 12 egg chambers by qPCR also indicated that amplicon origin activity was severely inhibited (Supplemental Figure S4). Nonetheless, the level of acetylation on H4K12, H4K16, and H3K56 at DAFC-66D was similar between the *UAS:dap*–expressing and wild-type control cells (Figure 5). These results suggest that the majority of acetylation does not depend on origin activation and therefore does not correspond to nucleosome assembly behind the replication fork.

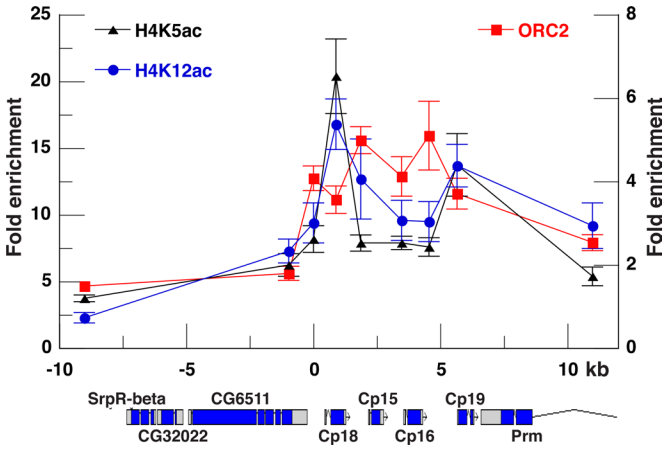


FIGURE 6: ORC binding and histone acetylation occur in a similar extended domain around the DAFC-66D origin. ChIP-qPCR analysis of Orc2 binding (red square, right *y*-axis) over an ~20-kb region surrounding the DAFC-66D origin in follicle cells from stage 10. H4K5ac (black triangle, left *y*-axis) and H4K8ac (blue circle, left *y*-axis) data for the 7 kb around DAFC-66D were taken from Figure 2. Other types of acetylation gave a similar profile over this 20-kb domain but are not shown for simplicity. The error bars represent the range of data from two or three biological replicates.

### ORC binds in an extended domain at DAFC-66D with a profile that resembles acetylation

We next determined the relationship between acetylation and binding of the ORC to origin DNA, a prerequisite for subsequent assembly of the preRC. It was previously reported that Ori-β and ACE3 are preferred binding sites for the ORC in vitro and in vivo (Austin *et al.*, 1999). ChIP-qPCR with antibodies against the Orc2 subunit, however, revealed that ORC is significantly enriched in an extended domain around the DAFC-66D locus (Figure 6). We extended the analysis of ORC binding and acetylation by using primer pairs −10 and +10 kb from ACE3, which indicated that both Orc2 and acetylation of multiple lysine residues are at least twofold enriched at these sites over a control locus (Figure 6). The profile of ORC binding in this 20-kb domain was strikingly similar to that of acetylation, except that ORC occupancy did not display a sharp peak of enrichment adjacent to Ori-β. These data indicate that ORC binds and nucleosomes are acetylated in an extended domain around DAFC-66D in vivo and are not restricted to the essential origin regions ACE3 and Ori-β.

### Acetylation on some histone lysine residues depends on ORC

The similarity in the profile for nucleosome hyperacetylation and ORC binding raised the possibility that they may have a functional relationship to one another. In fact, previous data showed that altering acetylation levels influences the selection of ORC binding sites and active origins in follicle cells (Aggarwal and Calvi, 2004; Lewis *et al.*, 2004). We could not easily test how mutation of different histone lysines affected preRC assembly and origin activity because there are hundreds of copies of histone genes in *Drosophila*, making such an analysis difficult. We could address, however, whether preRC assembly affected acetylation by using genetics to block ORC binding in vivo and then analyzing acetylation by ChIP-qPCR. Two mutations in the Orc6 subunit, Orc6$^{K76A}$ and Orc6$^{S72A}$, impair

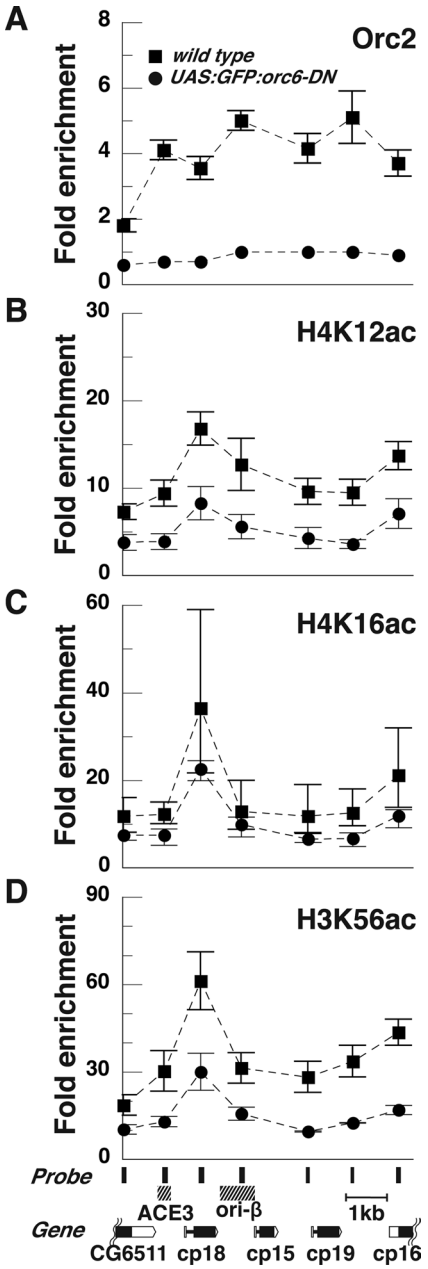


FIGURE 7: Acetylation of H4K12 and H3K56 depends on ORC binding. (A) ChIP-qPCR results for DAFC-66D using α-Orc2 antibodies on stage 10 follicle cells from wild-type Oregon R (■) and *c323GAL4/+;UAS:orc6$^{S72A}$/+* (●) flies. (B–D) ChIP-qPCR results using the indicated histone acetylation antibodies on stage 10 follicle cells from wild-type Oregon R (■) and *c323GAL4/+;UAS:orc6$^{K76A}$/+* (●) flies. The Oregon R data from Figure 2 are graphed in B–D for comparison. Drawn to scale for the DAFC-66D map shown below. The error bars represent the range of data from two or three biological replicates.

binding of the ORC to ACE3 and Ori-β DNA in vitro (Balasov *et al.*, 2007). We found that expression of *UAS:GFP:orc6$^{K76A}$* or *UAS:GFP:orc6$^{S72A}$* (flies provided by I. Chesnokov) using the c323GAL4 driver partially inhibited amplification. Although most follicle cells had detectable BrdU foci, the fluorescence intensity of these foci was diminished, and females produced eggs with thin shells (data not shown). Quantification of DNA copy number by qPCR in stage 10 and stage 12 follicle cells also showed that amplification was inhibited in *UAS:GFP:orc6$^{K76A}$*–and *UAS:GFP:orc6$^{S72A}$*–expressing flies (Supplemental Figure S4). To evaluate whether the inhibition of amplification was due to reduced ORC binding to DNA in vivo, we conducted ChIP with anti-Orc2 antibodies (Gossen *et al.*, 1995; Royzman *et al.*, 1999). In *UAS:GFP:orc6$^{S72A}$*–expressing follicle cells, ORC binding at DAFC-66D was not detectable across the entire locus (Figure 7A). As a control, we also expressed a wild-type *UAS:GFP:orc6*. To our surprise, this also inhibited ORC binding and amplification at DAFC-66D (Supplemental Figures S4 and S5). Although we do not understand the molecular basis for this, one possibility is that the green fluorescent protein (GFP) fusions on all these Orc6 proteins poisons the six-subunit ORC and disrupts origin binding. Nevertheless, we can use these transgenes as a tool to disrupt ORC binding to DNA and evaluate its effect on nucleosome acetylation.

Analysis of acetylation in *orc6$^{K76A}$*-expressing cells indicated that both H4K12ac and H3K56ac levels were reduced at DAFC-66D (Figure 7, B and D). The variance for H4K16ac in wild type, however, was too high for us to confidently conclude how this modification was affected by ORC binding (Figure 7C). These results suggest that at least H4K12ac and H3K56ac partially depend on ORC binding to DNA.

We next blocked the downstream preRC assembly step of MCM helicase loading by expressing geminin, an inhibitor of Cdt1 (Wohlschlegel *et al.*, 2000; Quinn *et al.*, 2001; Tada *et al.*, 2001). Expression of *UAS:gem* in follicle cells severely reduced BrdU incorporation at amplification foci, and females produced eggs with thin shells. BrdU incorporation was mosaic in egg chambers, with only 18 ± 9% of cells (n = 225) having detectable BrdU incorporation. qPCR quantification of amplicon copy number in stages 10 and 12 also showed that *UAS:gem* inhibited amplification (Supplemental Figure S4). Unlike the UAS:*orc6* cells, *UAS:gem* cells had normal levels of H4K12ac (Figure 8A). This suggests that H4K12ac depends on ORC binding but is independent of the downstream MCM helicase-loading step. Again, the variance for H4K16ac was too high among replicates to confidently conclude how MCM loading influences this modification (Figure 8B). Parallel ChIP of the same samples with antibodies against H3K56ac indicated that this modification was increased in the *UAS:gem*–expressing cells (Figure 8C). Combined, the data indicate that H4K12ac is dependent on ORC but independent of the downstream step of MCM loading. In contrast, although H3K56ac also depends on ORC binding, it becomes hyperacetylated when MCM loading is impaired.

## DISCUSSION

We used the gene amplification model system to investigate the epigenetic regulation of origins during development. Previous data suggested that nucleosome acetylation contributes to specifying active amplicon origins in follicle cells. Our present data indicate that multiple histone lysines are hyperacetylated when the origin is activated and that all these acetylation marks rapidly decline later when the origin shuts off and nearby promoters turn on. Although nonorigin loci had similar types of acetylation, our data reveal a quantitative relationship between the level of acetylation and origin activity. Together with previous results, the data suggest that hyperacetylation on multiple histone lysines may contribute to origin locus specificity and developmental timing. Our data also provide a higher-resolution picture of the origin epigenetic landscape and show that nucleosomes are hyperacetylated and ORC binds in an extended domain around DAFC-66D, with a peak of acetylation adjacent to Ori-β, the major site of initiation. The most surprising finding, however, was that acetylation on some lysines

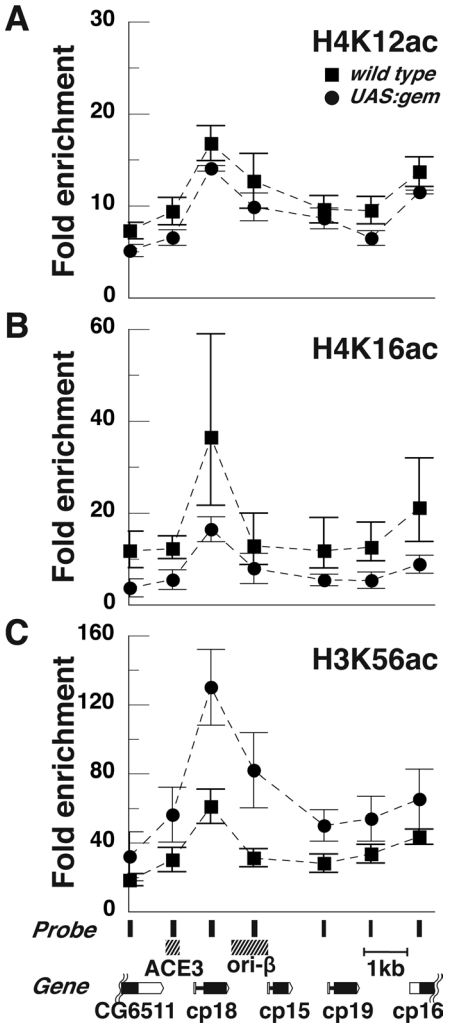


FIGURE 8: Inhibition of MCM loading does not affect acetylation of H4K12 but increases acetylation on H3K56. Comparison of acetylation level at DAFC-66D between wild-type and *UAS:gem*–expressing follicle cells. (A–C) ChIP-qPCR results using the indicated antibodies on stage 10 follicle cells from wild-type Oregon R (■) and *c323GAL4/+;UAS:gem/+* (●) flies. The Oregon R data from Figure 2 were graphed for comparison. Drawn to scale for the DAFC-66D locus shown below. The error bars represent the range of data from two or three biological replicates.

depends on ORC binding to the origin, suggesting that multiple HATs may be recruited during origin licensing. These results have important and general implications for understanding origin regulation and the coordination of origin and promoter activity during development.

### An epigenetic switch model for developmental gene amplification

Our results provide new insights into the relationship between histone acetylation and the activity of amplicon origins. Previous data indicated that mutation of the general HDAC Rpd3 results in genome-wide hyperacetylation, ORC binding, and genomic replication in stage 10B follicle cells rather than the normal site-specific amplification (Aggarwal and Calvi, 2004; Lewis *et al*., 2004). That result suggested that acetylation of nucleosomes contributes to origin specification in follicle cells. We also developed a DAFC-66D origin reporter and used it to show that tethering the *Drosophila* orthologues of the HAT HBO1 or HDAC Rpd3 to the origin in vivo increased or decreased amplification level, respectively (Aggarwal and Calvi, 2004). Recent results from the Orr-Weaver lab using this reporter confirm that tethering the Rpd3 HDAC represses the origin and further show that acetylation of H4K8 in the reporter is decreased (Kim *et al*., 2011). Results from multiple labs therefore suggest that nucleosome acetylation contributes to amplicon origin efficiency and locus specificity (Hartl *et al*., 2007). It was not previously known, however, what spectrum of acetylation types occurs at the origin, nor how these different nucleosome modifications coincided with temporal activation and repression of the origin. Our present data with validated antibodies show that nucleosome hyperacetylation at the origins occurs on multiple histone lysines. From our immunofluorescence and ChIP data, however, it is clear that acetylation on these same lysines occurs at some nonorigin loci in stage 10 follicle cells. This suggests that nucleosome acetylation is not sufficient to specify active origins. We found, however, that the relative level of acetylation is much higher at origin loci and that there is a quantitative correlation between the level of hyperacetylation and the number of times different amplicon origins initiate replication. Recent follicle cell array data from the Orr-Weaver lab also show that histone acetylation is not unique to the active amplicons but that there is a quantitative relationship between the level of hyperacetylation and amplicon origin activity (Kim *et al*., 2011). The current evidence therefore fails to support a qualitative histone code for amplicon origins, but instead reveals a quantitative correlation between the level of nucleosome hyperacetylation and origin activity.

Our data show a temporal correlation between hyperacetylation on multiple lysines and amplicon origin activity during oogenesis. Multiple acetylation marks rapidly increase when the origins become active and then rapidly decline when the origin shuts off. Rapid decline of acetylation at the DAFC-66D origin in stage 12 correlates with the departure of ORC from the origin and is followed by activation of nearby chorion protein promoters (Shea *et al*., 1990; Austin *et al*., 1999; Royzman *et al*., 1999; Aggarwal and Calvi, 2004). Together with our previous evidence, our data lead us to propose an epigenetic switch model in which dynamic acetylation–deacetylation on multiple lysines participates in the locus specificity, efficiency, and developmental timing of amplicon origins and coordinates sequential origin and promoter activity (Figure 9). Although the mechanistic details of this model have yet to be worked out, it makes testable predictions about the epigenetic regulation of origins and the coordination of origin and promoter activity.

### Acetylation depends on preRC assembly

Our most surprising result was that acetylation on different histone lysines depends on ORC. Evidence from yeast and metazoa supports the notion that chromatin can influence both the site of preRC assembly and the time of preRC activation during S phase (Mechali, 2010). Our results, however, indicate that the cause and effect can operate in the opposite direction as well, and that preRC assembly influences chromatin modification. Although nucleosome acetylation was not affected when origin activation was inhibited by dacapo, acetylation was reduced on H4K12 and H3K56 when ORC function was disrupted. Our data showed that reduced ORC binding to the origin correlated with a local reduction in nucleosome acetylation in three genotypes and six biological replicates. It remains possible, however, that the reduced acetylation is caused by an unknown indirect effect of ORC overexpression rather than a disruption of ORC binding per se. Nonetheless, H4K12ac was clearly dependent on ORC, but it was not changed when MCM loading was

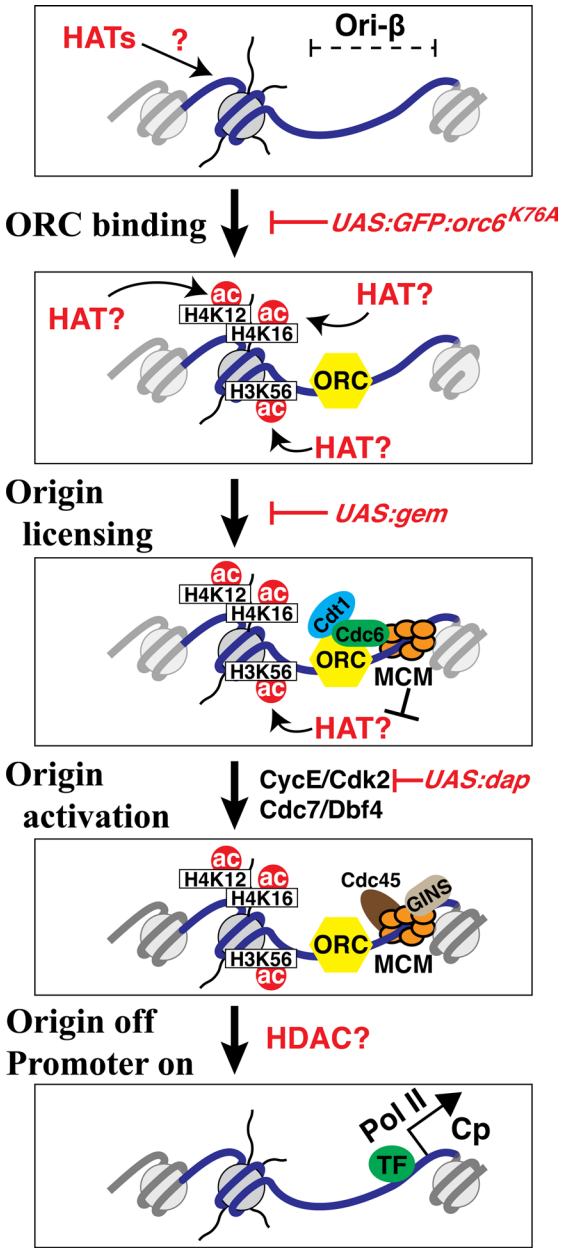


FIGURE 9: An epigenetic switch model for nucleosome acetylation and origin activity at the DAFC-66D origin. This model illustrates steps of origin specification (initial ORC binding), licensing (loading of MCMs), and origin activation by CDK2 and CDC7 at DAFC-66D. Other replication proteins that associate with the origin are not shown. Genetic manipulations that we used to inhibit these three steps are shown in red italics between the boxes. The drawing shows only one ORC bound to Ori-β and the nucleosome next to it for visual clarity, but our data indicate that both ORC binds and nucleosomes are acetylated in an extended domain that encompasses the origin. Multiple lysines are acetylated, suggesting that multiple HATs participate in origin regulation. H4K12ac and H3K56ac were decreased when ORC binding was inhibited, suggesting that the HATs responsible for these modifications may be recruited or activated, depending on ORC. H3K56 acetylation increased when origin licensing was inhibited, suggesting a possible negative feedback loop between MCMs and the H3K56 HAT (black crossbar in box 3). The last step represents rapid deacetylation of the origin by unknown HDACs in stage 12, which is associated with the departure of ORC, origin silencing, and activation of nearby chorion protein (cp) promoters by transcription factors (TF). See the text for further details.

inhibited. This suggests that the HAT responsible for H4K12ac may be recruited or activated after ORC binding but before the MCM loading step of preRC assembly. H3K56ac was also dependent on ORC but was hyperacetylated when the MCM loading step was inhibited. One possibility is that the HAT for H3K56 is also recruited by ORC to promote loading of MCM helicase rings onto DNA and that a negative feedback mechanism inhibits this HAT or activates an HDAC responsible for regulating acetylation of H3K56 once sufficient MCM helicase complexes are loaded. An important prediction from these results is that multiple HATs may be recruited or activated during different steps of preRC assembly and may contribute to origin licensing. The activation and repression of origins therefore may be analogous to that of promoters, in which codependent recruitment of multiple chromatin modifiers and transcription factors determines the developmental specificity of transcription.

### HATs and HDACs in origin regulation

Our results have parallels to growing evidence from yeast to humans that suggests that HATs and HDACs influence origin identity, efficiency, and timing during genomic DNA replication. We previously showed that GAL4 fusions of the fly orthologue of HBO1 could stimulate the DAFC-66D origin when tethered to it (Aggarwal and Calvi, 2004). In human cells, HBO1 is recruited to origins by Cdt1 and is required for MCM loading (Miotto and Struhl, 2010). Evidence suggests that HBO1 acetylates H4K5, H4K8, and H4K12, all modifications that we find at the amplicon origins, but cannot explain the acetylation of H3 tails, H4K16, or H3K56 (Doyon *et al.*, 2006; Miotto and Struhl, 2010). Two candidate HATs for these modifications are CBP, which has been shown to acetylate H3K56 in multicellular eukaryotes, and MOF, which acetylates H4K16 to increase transcription from the *Drosophila* male X chromosome during dosage compensation (Kelley *et al.*, 1999; Das *et al.*, 2009; Gelbart and Kuroda, 2009; Kharchenko *et al.*, 2011). In *Drosophila* tissue culture cells, acetylation of H4K16 by MOF confers early replication to the male X chromosome, and genomic sites of ORC binding and active origins correlate with enrichment for H4K16Ac and other acetylation marks (Schwaiger *et al.*, 2009; Eaton *et al.*, 2010b). Our data are also consistent with a report that nucleosomes at origins in *Saccharomyces cerevisiae* are acetylated on multiple lysine residues, including those that we found at the amplicon origins (Unnikrishnan *et al.*, 2010). Finding multiple, similar acetylation marks at yeast and *Drosophila* origins suggests that the regulation of DNA replication by multiple HATs may be conserved in eukaryotes.

The rapid decline in acetylation in stage 12 suggests that the action of multiple HATs at the origin may be counteracted by multiple HDACs. Our previous data showed that Rpd3 is required to repress nonamplicon origins in stage 10B follicle cells (Aggarwal and Calvi, 2004). In yeast, the HDAC Rpd3 influences the time of origin activation, whereas the HDAC Sir2 can regulate the location of preRC assembly (Vogelauer *et al.*, 2002; Crampton *et al.*, 2008; Fox and Weinreich, 2008). *Drosophila* Sir2 can deacetylate H3K56, and therefore our results raise the possibility that this HDAC also regulates origins in metazoa (Das *et al.*, 2009). It was also recently shown that HDAC11 counteracts acetylation by HBO1 to regulate DNA replication in Chinese hamster ovary cells (Wong *et al.*, 2010). An important future goal, therefore, is to further define how these and other HATs and HDACs are recruited to and directly regulate amplicon and other origins.

### The origin epigenetic landscape

A number of origins in metazoa have been analyzed molecularly and genetically, but the picture for origin anatomy and function

remains fuzzy at best. Our analysis of DAFC-66D locus has begun to provide a fine-scale picture of the epigenetic landscape of this well-characterized model origin. Previous reports indicated that the ACE3 and Ori-β regions of DAFC-66D are preferred ORC binding sites, are evolutionarily conserved, and are required in *cis* for amplification (Delidakis and Kafatos, 1989; Orr-Weaver *et al.*, 1989; Austin *et al.*, 1999; Remus *et al.*, 2004; Zhang and Tower, 2004; Claycomb and Orr-Weaver, 2005; Calvi, 2006; Calvi *et al.*, 2007). We found that nucleosome hyperacetylation was not restricted to ACE3 and Ori-β but was spread over the entire 20 kb that we analyzed. Of importance, ORC occupancy was also enriched across the entire hyperacetylated region with a similar profile. Recent array data from the Orr-Weaver lab also showed that H4K8 is hyperacetylated and ORC binds in an extended genomic domain of ~30 kb around DAFC-66D and in smaller domains around other amplicons, consistent with evidence from Mike Botchan's laboratory (Kim *et al.*, 2011; M. Botchan, personal communication). The concordance between nucleosome acetylation and ORC binding profiles is consistent with a functional relationship between them. Our genetic data showing that at least some acetylation marks depend on ORC binding opens the possibility that binding of multiple ORC complexes may promote an extended domain of nucleosome acetylation. At present we do not know whether this extended ORC domain represents binding of ORC to different sites on different DNA strands or binding of multiple ORCs on the same DNA strand. The possibility that multiple ORCs bind a single DNA strand is consistent with previous evidence for increased ORC occupancy on longer origin fragments in vitro and the binding of multiple ORCs at some origins in yeast and metazoa in vivo (Bielinsky *et al.*, 2001; Takahashi *et al.*, 2003; Bolon and Bielinsky, 2006; Aladjem, 2007). However, the functional significance of multiple ORCs for origin activity or initiation site selection remains unclear. One possibility is that binding of ORC to its preferred sites in ACE3 and/or Ori-β promotes subsequent recruitment and spreading of additional ORCs, analogous to spreading of multiple DnaA complexes at Ori-C in *Escherichia coli* (Clarey *et al.*, 2006; Erzberger *et al.*, 2006; Remus and Diffley, 2009).

Another fine-scale attribute of the DAFC-66D epigenetic landscape was that all acetylation marks that we examined were highest to one side of Ori-β, in the 3′ end of the cp18 gene, which is not expressed when the origin is active. This suggests that among the population of chromatin fibers, acetylation occurs more frequently on nucleosomes to one side of Ori-β, the site where replication initiates 80% of the time (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). A peak of acetylation was also observed at DAFC-7F within ACE1, a region that binds ORC and is essential for origin function. In *S. cerevisiae*, nucleosomes positioned adjacent to some origins promote ORC binding, likely through interaction with the bromo adjacent homology (BAH) domain of Orc1, a domain that is also important for the binding of human Orc1 to chromatin and mutation of which causes the developmental abnormality Meier–Gorlin syndrome (Lipford and Bell, 2001; Noguchi *et al.*, 2006; Muller *et al.*, 2010; Bicknell *et al.*, 2011; Guernsey *et al.*, 2011). The peak of acetylation adjacent to the major initiation site in Ori-β may therefore reflect a positioned nucleosome that promotes initial ORC binding or other steps of origin licensing and activation. Further analysis of nucleosome position, nucleosome modification, and preRC occupancy at DAFC-66D will permit us to test this model and dissect origin anatomy and function with high resolution.

## MATERIALS AND METHODS

### *Drosophila* strains

The Oregon R$^{modENCODE}$ strain was used as the reference wild-type strain. C323:GAL4 was used to drive expression of *P{w$^+$UAS:GFP:orc6$^{K76A}$}*, *P{w$^+$UAS:GFP:orc6$^{S72A}$}*, and *P{w$^+$UAS:GFP:orc6}* (gifts from Igor Chesnokov, University of Alabama at Birmingham, Birmingham, AL; Balasov *et al.*, 2007), *P{w$^+$UAS:dacapo}* (Lane *et al.*, 1996), and *P{w$^+$UAS:geminin}* (this study).

### Antibodies

α-BrdU (mouse monoclonal, BDB347580; BD Biosciences, San Diego, CA), α-H4K5ac (07-327; Upstate, Millipore, Billerica, MA), α-H4K8ac (07-328; Upstate), α-H4K12ac (39165; Active Motif, Carlsbad, CA), α-H4K16ac (39167; Active Motif), α-H3K56ac (07-677; Upstate), α–histone H3 C-terminal (39163; Active Motif), H3K4me3 (39159; Active Motif), α-H3K27me3 (07-449; Upstate), α-H3K9/14ac (06-599; Upstate), α-Orc2 (gift of Stephen Bell, MIT, Cambridge, MA; Austin *et al.*, 1999), and α-Orc2 (gift of Michael Botchan, University of California, Berkeley, CA). Except where stated, all antibodies were rabbit polyclonals.

### Immunostaining and microscopy

BrdU and antibody labeling of *Drosophila* ovaries was as previously described (Calvi *et al.*, 1998; Calvi and Lilly, 2004). Antibody concentrations were as follows: α-BrdU (1:20), α-H4K12ac (1:100), and α-H4K16ac (1:100). Images are Z-stack projections taken with a Leica (Wetzlar, Germany) SP5 scanning confocal microscope.

### Follicle cell nuclear preparation for ChIP

Follicle cell nuclei were purified from different stage egg chambers based on a modification of several protocols (Petri *et al.*, 1976; Woll *et al.*, 1981). Females were conditioned with males on wet yeast for 3 d and then blended by short pulses in cold phosphate-buffered saline buffer in 0.02% Tween-20 in a household blender. Released egg chambers were enriched by serial filtration through 250- to 70-µm meshes and repeated resuspension–resettling in cold buffer. Eggs were then fixed for 15 min at room temperature in 2% paraformaldehyde solution, followed by fix quenching with 125 mM glycine and washing with cold Dulbecco's phosphate-buffered saline. Stage 10, stage 12, and stage ≤8 egg chambers were then further manually separated and stored at −80°C prior to nuclear preparation. Approximately 4000 stage 10 and ~3000 stage 12 egg chambers were used for nuclear preparation. The number of stage ≤8 egg chambers was not counted, but a tissue volume similar to that of the other stages was used. Frozen eggs were thawed on ice, resuspended in mHB buffer (0.34 M sucrose, 15 mM NaCl, 60 mM KCl, 0.2 mM EDTA, 0.2 mM ethylene glycol tetraacetic acid, 0.15 mM spermine, 0.15 mM spermidine in 15 mM Tris-HCl, pH 8.0) supplemented with 0.5% NP-40 and transferred to a Kontes 2-ml douncer. Fifteen strokes with a type A pestle were applied, and the content was filtrated with a 15-µm Nitex nylon membrane to remove the larger nurse cell nuclei. The filtrate was spun 3 min at 500 × *g* in a microcentrifuge to pellet follicle nuclei.

### Chromatin immunoprecipitation

The ChIP protocol was modified from previous methods (17-295; Millipore) and entailed at least two biological replicates from separate isolations of follicle cells. In brief, prepared follicle nuclei were resuspended in nuclear lysis buffer (1% SDS, 1 mM EDTA in 50 mM Tris-Cl, pH 8.0) and subjected to sonication (Fisher dismembrator model 100 [Thermo Fisher Scientific, Waltham, MA], tip probe, setting 2, five rounds of 20-s sonication on ice) to a modal size of 450

base pairs. One-tenth of the sample was saved as input, and the remainder was aliquoted and diluted at least fivefold into binding buffer (0.01% SDS, 1.1% Triton-X 100, 1.1 mM EDTA, 167 mM NaCl in 20 mM Tris-Cl, pH 8.0) to a final volume of 1 ml. Immunoprecipitation was then performed in parallel by adding 2 µl (~2 µg) of antibodies, followed by nutation at 4°C overnight. The next day 30 µl of 50% protein A agarose beads (15918-014; Invitrogen) pretreated with sheared salmon sperm DNA was added and incubated 1 h at 4°C and precipitated by brief centrifugation. The beads were washed two times each with low-salt, high-salt, LiCl, and Tris–EDTA buffers. Elution and reversal of cross-linking was done in nuclear lysis buffer with heat at 65°C overnight. Both the input and eluate were treated with 1 µg of RNaseA (11119915001; Roche, Indianapolis, IN) and 50 µg of proteinase K (P8102S; New England BioLabs, Ipswich, MA). DNA was then purified with standard phenol/chloroform procedure and used for qPCR analysis.

### qPCR

Analysis was done on a Stratagene (Santa Clara, CA) Mx3005P machine with SYBR Green Master Mix (600843; Agilent, Santa Clara, CA). Forty cycles of PCR were run with a two-step protocol (denaturation at 95°C for 15 s and annealing/extension at 62°C for 30 s). For ChIP-qPCR experiments, the amount of DNA in the pellet was expressed as percentage of input DNA estimated by a standard curve generated from a serial dilution of the input. The values were then normalized to a control, nonorigin locus at cytogenetic position 64A. Normalization to another, nonamplified region at 93E/F gave similar results. To measure developmentally amplified DNA copy number in wild-type and transgene-expressing flies, the relative copy numbers at different loci were calculated by ΔCt from a reference locus at cytogenetic region 93E/F. PCR primer sequences are available upon request.

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